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Clostridium botulinum and its neurotoxins: a metabolic and cellular perspective

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Abstract

Clostridium botulinum comprises a diverse assemblage of clostridia that have the common property of producing a distinctive protein neurotoxin (BoNT) of similar pharmacological activity and extraordinary potency. BoNTs are produced in culture as molecular complexes consisting of BoNT, hemagglutinin (HA) and associated subcomponent proteins, nontoxic nonhemagglutinin (NTNH), and RNA. The genes encoding the protein components reside as a cluster on the chromosome, on bacteriophages, or on plasmids depending on the *C. botulinum* serotype. A gene *BotR* coding for a regulatory protein has been detected in toxin gene clusters from certain strains, as well as ORFs coding for uncharacterized components. The gene encoding TeNT is located on a large plasmid, and expression of the structural gene is controlled by the regulatory gene, *TetR*, located immediately upstream of the TeNT structural gene. TeNT is not known to be assembled into a protein/nucleic acid complex in culture. Cellular synthesis of BoNT and TeNT have been demonstrated to be positively regulated by the homologous proteins, BotR/A and TetR. Evidence suggests that negative regulatory factors and general control cascades such as those involved in nitrogen regulation and carbon catabolite repression also regulate synthesis of BoNTs. Neurotoxigenic clostridia have attracted considerable attention from scientists and clinicians during the past decade, and many excellent reviews are available on various aspects of these organisms and their neurotoxins. However, certain areas have not been well-studied, including metabolic regulation of toxin formation and genetic tools to study neurotoxigenic clostridia. These topics are the focus of this review.

1. Introduction

Clostridium botulinum and *Clostridium tetani* have attracted much interest in recent years due largely to extensive research on the biochemistry, structure, pharmacology, and cell biology of their neurotoxins (BoNTs and TeNT) (Bigalke and Shoer, 2000; Kreydon et al., 2000; Rosetto et al., 2001; Schiavo et al., 2000), as well as the application of botulinum toxin complexes as therapeutic agents for the treatment of human diseases (Brin, 1997; Dressler, 2000; Jankovic and Hallett, 1994). Several hundred research papers have been published in these areas, while relatively few studies have addressed the physiology and genetics of neurotoxigenic clostridia and the regulation of neurotoxin formation.

Early interest in the molecular biology of clostridia mainly involved studies of metabolism and physiology of nonpathogenic clostridia such as solvent-producing species (e.g. *C. acetobutylicum*), clostridia producing extracellular hydrolytic enzymes or other products of industrial interest, nitrogen-fixing species, those forming lethal toxins, and species with perplexing degradative pathways for amino acids and other nitrogenous compounds (Andreesen et al., 1989; Barker, 1978; Johnson, 1999; Minton and Clarke, 1989; van Heyningen, 1950). Kato (1970) addressed the relation of metabolism and cell structure to toxin production, but this subject has not been reviewed recently for the neurotoxigenic clostridia. The necessity to produce vaccines for prevention of clostridial human and animal diseases necessitated study of environmental and nutritional factors to increase toxin production, and methods to isolate the toxic fractions from culture broths (Brown and Williamson, 1997; Johnson & Goodnough, 1995; Schantz and Johnson, 1992). Early studies on toxin production and isolation were later complemented by characterization of plasmids, bacteriophages, bacteriocins, and the isolation of mutants in

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clostridia (Eklund et al., 1989; Sebald, 1993; Young et al., 1989). Beginning in the 1980's, various genes were cloned from clostridia, and the structure of genetic elements and mechanisms involved in the regulation of transcription and translation began to be studied (Young et al., 1989; Minton, 1995; Rabinowitz, 1993; Sebald, 1993). The cloning, expression and sequencing of genes encoding toxins led to the field of molecular toxinology (Alouf, 1988; Niemann, 1991; Sebald, 1993). The detection of toxin genes on plasmids and bacteriophages in neurotoxigenic clostridia (Inoue and Iida, 1970; Eklund et al., 1971) was followed by the demonstration of lateral transfer of toxin genes in *C. botulinum* serotypes C and D and *C. novyi* (Eklund et al., 1989; Eklund, 1993). The findings that certain *C. botulinum* isolates produce more than one serotype of toxin or contain silent (unexpressed) gene clusters supported the lateral transfer of genes encoding neurotoxigenicity (Gimenez and Ciccarelli, 1970; Franciosa et al., 1994; Hatheway and McCroskey, 1987; Hutson et al., 1996). Toxin gene transfer also was suspected from investigations of intestinal botulism cases, which showed that type E and F BoNT genes were present in rare strains of normally nontoxigenic clostridial species (Aureli et al., 1986; Hall et al., 1985; McCroskey, et al., 1986, 1991). Despite these observations and their importance to toxinology and medicine, the molecular mechanisms governing toxin synthesis and interspecies transfer of the toxin genes remain largely unknown. Genetic tools for study of these mechanisms are in early stages of development. Genetic manipulation of neurotoxigenic clostridia has developed slowly compared to other clostridia such as *C. perfringens* and *C. acetobutylicum* (Lyras and Rood, 2000).

2. Clostridium botulinum—the organism

As presently defined, the genus *Clostridium* consists of a phenotypically and phylogenetically diverse assemblage of Eubacteria that have four common phenotypic properties: (a) the formation of heat- and chemical-resistant endospores; (b) the presence of a Gram-positive cell wall structure in vegetative cells; (c) an anaerobic, fermentative metabolism and; (d) a low guanine + cytosine (G + C) content of 38–56 mol% (26–32% for toxigenic species) (Cato et al., 1986; Hippe et al., 1999). Members of the genus *Clostridium* are extremely prolific in making toxins, and the clostridia produce more extracellular protein toxins than any other bacterial genus (Hatheway, 1990; Hatheway and Johnson, 1998; van Heyningen, 1950). *C. botulinum* produces seven serotypes of BoNTs, designated A to G. The toxigenic clostridia are not a newly discovered group of bacteria and were initially recognized in 1877 by Pasteur in collaboration with Joubert, who cultivated for the first time a toxigenic anaerobic bacterium, *Clostridium septicum* (Sebald and Hauser, 1995). The type species of the genus, *Clostridium butyricum* ('Vibrion butyrique'), was probably also isolated in pure culture by Pasteur in 1861 (Hatheway and Johnson, 1998; Sebald and Hauser, 1995).

The most common habitats of clostridia are the soil (Dodds, 1993; Popoff, 1992; Smith, 1955) and the feces of humans and animals (George and Finegold, 1985; Hatheway and Johnson, 1998), but the spores are resistant to environmental stresses and are also found in various foods, sewage, and other environments. Unlike many other clostridial species, *C. botulinum* spores are rarely found in human feces unless the individuals have contracted botulism, and coproexamination has been suggested as a partial diagnosis of botulism (Dowell et al., 1977).

The taxonomy of the clostridia has traditionally been determined by cultural properties, morphological characters including the vegetative rod-shape and production of endospores, phenotypic properties including fermentation patterns of carbohydrates and nitrogenous substrates, volatile acid formation, cell wall structure, and genetic methods such as determination of DNA–DNA hybridization, 16S rRNA sequence homology, and mol% G + C content. Several treatises have described these traditional methods for isolation, cultivation, characterization of *Clostridium* (Cato et al., 1986; Hippe et al., 1999; Holdeman et al., 1977; Summanen et al., 1993; Willis, 1969a,b; 1977). Beginning in the 1970s (Johnson and Francis, 1975) and becoming increasingly popular in recent years, the traditional phenotypic methods of identification have been complemented by PCR amplification of target gene regions (usually 16S rRNA regions or intergenic spacer sequences between 16S and 23S rRNA genes) and determination of the relatedness of the amplified nucleotide sequences (Collins et al., 1994; Collins and East, 1998; Johnson, 1984). By this approach, it has been confirmed that *Clostridium* is a highly polyphyletic genus, and it has been proposed to consist of more than 20 genera and several families (Collins et al., 1994; Stackenbrandt and Rainey, 1997). Sequencing of the genes encoding 16S rRNA is undoubtedly a valuable technique to assess phylogenetic relationships of organisms, but when used exclusively for classification it may leave gaps in our understanding of the toxigenic clostridia, particularly in areas of physiology, regulation of toxin production, toxin gene transfer, and development of enrichment and detection methods for discovery of new neurotoxigenic clostridia. Classification of clostridia by a polyphasic approach (Vandamme et al., 1996) that combines phenotypic and genotypic properties provides a comprehensive characterization.

Eventually, the complete nucleotide sequencing of the genomes of toxigenic clostridia will provide the most detailed insights into the biology of these organisms as it has in others (Allsop, 1998; Anonymous, 2000; Broder and Venter, 2000; Strauss and Falkow, 1997). It is humbling to realize that the complete sequence of a microbial genome (*Haemophilus influenzae* Rd) was obtained only about 6 years ago (Fleischmann et al., 1995), but now more than 30 genomic sequences are publicly available (Broder and

Venter, 2000), and more than 60 will be available in the near future (Anonymous, 2000). The sequencing of several *Clostridium* genomes is reportedly underway including those of *C. difficile*, *C. perfringens*, *C. botulinum* (type A and nonproteolytic B), and *C. acetobutylicum*/*C. beijerinckii*.

3. Neurotoxin and antibody detection

Irrespective of their taxonomic affiliations, the defining character of neurotoxigenic clostridia is the synthesis of a large neurotoxic protein of ~150 kDa with remarkable neuronal specificity (Schiavo et al., 2000) and extraordinary potency (Gill, 1982; Sugiyama, 1980). The toxicity of BoNT/A has been estimated to be ~0.2 ng/kg body weight, and as little as 0.1–1 μg may be lethal for humans (Schantz and Johnson, 1992; Scott and Suzuki, 1988). Consequently, considerable care and safety precautions are needed in working with neurotoxigenic clostridia and NTs. The Centers for Disease Control and Prevention (CDC) recommends Biosafety Level 3 primary containment and personnel precautions for laboratories producing large (milligram) quantities of the toxins (Centers for Disease Control and Prevention, 1998). Safety precautions have been briefly outlined in reviews (Malizio et al., 2000; Shone and Tranter, 1995).

Since NTs are the defining character of neurotoxigenic clostridia, carefully-conducted and validated methods are needed for detection and quantitation of neurotoxicity. Detection of BoNT is typically determined by intraperitoneal (ip) injection of mice together with neutralization with type-specific antisera (Hatheway, 1988; Schantz and Kautter, 1978). Caveats and potential pitfalls in this assay have been described (Hatheway, 1988). Our laboratory commonly uses intravenous tail vein injection and monitoring of time to death to estimate toxicity (Boroff and Fleck, 1966).

Although the ip mouse bioassay has been much debated, particularly for comparison of toxin preparations in clinical use (Borodic et al., 1996; Brin, 1997; Van den Bergh and Lison, 1998), the quantal bioassay and mouse lethality remains the standard for detection and quantitation of in vivo neurotoxicity. Other methods of determining toxicity such as those determining immunological reactivity or catalytic activity need to be compared to the mouse bioassay before they are adopted for general use.

Various in vitro assays of BoNT and TeNT toxicity have been developed and those detecting immunological reactivity by ELISA, assaying catalytic activity, or determining effects on nerve cells in culture seem most promising for screening purposes in foods or in clinical samples (Hatheway and Ferreira, 1996; Pellizari et al., 1998; Soleilhac et al., 1996). These assays should also be valuable in the rational design of therapeutic inhibitors and detection of ligands that bind to NTs including investigations of the physiological receptor(s) (Herreros et al., 2000; Lalli et al., 1999). Bioassays in mice and ELISA formats have also been useful for detection of antibodies in clinical samples (Dressler et al., 2000; Hatheway and Dang, 1994). As epitopes are elucidated and immune recognition defined for the BoNTs (Atassi and Oshima, 1999), it may be feasible to develop predictive assays to detect primed T or B cells at early stages of the immune response by flow cytometry measurement of cytokines in blood samples incubated in the presence of toxin (Nomura et al., 2000). Since one of the major drawbacks to clinical use of botulinum toxin is the formation of antibodies (Borodic et al., 1996), such an assay detecting early events in the immune reponse could be very useful in managing antibody resistance in patients being treated with botulinum toxin preparations.

4. Disease states of neurotoxigenic clostridia and isolation of novel strains

The classic diseases of neurotoxigenic clostridia are tetanus wound infections followed by CNS intoxication and foodborne botulism with characteristic flaccid paralysis of motor and respiratory functions. Wound botulism, infant botulism, and intestinal botulism in non-infants were discovered in the 1940's, 1970's and 1980's respectively (Hatheway and Johnson, 1998). The first descriptions of the symptoms of tetanus were attributed to Hippocrates (Major, 1965; Montecucco, 1995) and those of botulism by Justinus Kerner (1786–1862) (Ergbuth and Naumann, 1999). In landmark investigations in the late 1800's, the causative agent of tetanus was demonstrated by Carle and Rattone in Italy and isolated in pure culture by Kitasato (Montecucco, 1995). The bacterial cause of type B botulism, 'Bacillus botulinus', and properties of the produced neurotoxin were described in the remarkable study of Van Ermengem (1897). The epidemiology, clinical symptoms of tetanus and botulism, and the different serotypes of *C. botulinum* causing disease have been thoroughly reviewed (Cherington, 1998; Finegold, 1998; Hatheway, 1995; Johnson and Goodnough, 1998) and are not repeated here.

Classically, *C. botulinum* has been classified into seven serotypes, designated A to G (Sugiyama, 1980). The different serotypes differ in pathogenicity for humans and animals (Popoff, 1992; Johnson, 2000; Smith and Sugiyama, 1988). In the 1970's and 80's strains of *C. botulinum* were isolated that produced more than one serotype of BoNT (Gimenez and Ciccarelli, 1970; Hatheway et al., 1981; Sugiyama et al., 1972). Generally, one of the BoNT serotypes was produced at much higher levels than the other (Sugiyama et al., 1972). Later in the mid-1990's it was found that many strains of *C. botulinum* contain silent (unexpressed) toxin gene clusters (Davis et al., 1995; Franciosa et al., 1994; Hutson et al., 1996).

During the past two decades, botulinum-like neurotoxins were detected in cultures of two normally nontoxigenic *Clostridium* sp., *C. baratii* and *C. butyricum*. The first

toxigenic strains were isolated from the stools of infants with botulism in Rome, Italy, and New Mexico, USA (Aureli et al., 1986; Hall et al., 1985; McCroskey et al., 1986). Hatheway's laboratory found that the isolates obtained from toxic enrichments did not resemble *C. botulinum* in several phenotypic characteristics, and through extensive cultural work and characterization they were able to define the neurotoxigenic strains to species. It should be emphasized that the initial isolation of these strains required methods and tests not normally performed for detection of *C. botulinum*. Also, the spores of these organisms are less heat-resistant than proteolytic *C. botulinum* strains normally responsible for infant botulism, and heat-treatments for spore enrichments and culture isolation should be performed at appropriate temperatures. Through the efforts of Hatheway and colleagues, it is anticipated that more species of *Clostridium* will be isolated that produce botulinum-like neurotoxins. Neurotoxigenic *C. butyricum* has recently been isolated from various geographical environments including China (Meng et al., 1997; Wang et al., 2000), India (Chaudry et al., 1998), and from additional infant botulism outbreaks in Italy (Fenicia et al., 1999). Also, intestinal botulism in adults has been observed, particularly in patients with underlying bowel diseases or who had recently undergone surgery and were receiving antimicrobial therapy (see Griffin et al., 1997 for reviews of cases).

Evidence has been found recently that clostridial colonization of the gastric and intestinal tracts of humans may precipitate diseases not formerly associated with clostridia (Sandler et al., 2000; Siarakas et al., 1999). An outstanding example is late-onset autism, a devastating behavioral disease beginning in childhood that was hypothesized to be caused by neurotoxin-producing clostridia in the gut (Bolte, 1998). Antibiotic therapy provided supporting evidence for this hypothesis (Sandler et al., 2000). The involvement of clostridia in autism suggests that other CNS-related ('mental') diseases may be caused by clostridial neurotoxins, and the potential for clostridial species to cause CNS disorders should be an intriguing area of research in the future.

5. The genome of *C. botulinum*

Initial studies of the genomes of toxigenic clostridia and generation of genetic maps were performed by pulsed-field gel electrophoresis (PFGE) and detection of markers on restriction fragments (Canard and Cole, 1989; Lin and Johnson, 1995). Our laboratory was the first to apply PFGE for genome analysis of *C. botulinum* type A (Lin, 1992. Thesis, University of Wisconsin–Madison; Lin and Johnson, 1995). The genomes of four group I (type A) strains examined had unique restriction digestion patterns, reflecting genotypic differences (Lin and Johnson, 1995). The genome size of *C. botulinum* strain 62A was estimated to be 4039 ± 40 kbp from the summation of restriction fragments generated by rare-cutting restriction enzymes. PFGE in *C. botulinum* is challenging since in certain strains good quality DNA is extremely difficult to obtain due to poor lysis of cells and high DNase activity. Unlike *C. perfringens* (Rood, 1998), only a few genes have been mapped to restriction fragments in *C. botulinum*. The genes encoding the toxin complex were mapped to a single restriction fragment in *C. botulinum* strain 62A and in Tn*916* mutants derived from this parent (Johnson et al., 1997; Lin and Johnson, 1991, 1995), indicating the physical linkage of the genes in the complex. In one of the tox$^-$ deletion mutants, the PFGE fragment containing the toxin gene cluster in the parent strain was reduced in size, and it was estimated that the Tn*916*-derived mutant contained a deletion of ca. 70 kb including the entire toxin gene region (Lin and Johnson, 1995).

PFGE was found to be a rapid and accurate method of determining genomic relationships between groups I and II of *C. botulinum* (Lin and Johnson, 1995; Hielm et al., 1998a). For group II *C. botulinum* serotype B, the genome size was estimated to be 3.6–4.1 kb (mean ± standard deviation = 3890 ± 170 kb). PFGE analyses have revealed extensive genetic diversity within *C. botulinum* type E strains examined (Hielm et al., 1998b). Genomic analysis of *C. botulinum* has also been investigated by other methods, including ribotyping, random amplified polymorphic DNA analysis (RAPD), and repetitive element sequence-based PCR (rep-PCR) (Hyytiä et al., 1999; Skinner et al., 2000). Generally, PFGE appears to be more discriminatory and consistent than most other methods for analysis of genomic variation. However, some other methods are more rapid than PFGE and can be used for strains for which satisfactory DNA preparations and restriction fragment digests cannot be obtained.

The coup de grâce in the genomic analysis of the toxigenic clostridia will be obtaining the complete nucleotide genomic sequences of selected strains. Genomic sequencing of certain clostridial species including group I (proteolytic) *C. botulinum* type A (Hall A), group II (nonproteolytic) *C. botulinum* type B strain (17B), *C. perfringens*, *C. difficile*, and *C. acetobutylicum* is underway in various laboratories (personal communications). The availability of complete genomic sequences should provide tremendous insight into the metabolism, genetics, toxigenesis, and evolution of the clostridia. However, due to the extensive genetic diversity of *C. botulinum*, even within a single serotype (Hielm et al., 1998b; Lin and Johnson, 1991), other approaches of genetic mapping should be used to complement genomic sequencing.

6. Nutritional regulation of BoNT and TeNT synthesis

Very little is known of the nutritional and environmental factors that influence BoNT and TeNT formation in media,

foods, wounds, and in the human gastric and intestinal tracts. Understanding the control of toxin synthesis and post-translational modification events such as nicking will be of considerable value in enhancing the quality and potency of toxin preparations. Early studies showed that supplementation of complex media with nutrients, such as meat digests, casein hydrolysates, corn steep liquor, calcium, glucose, individual amino acids, and other substances affected the synthesis of toxin in various serotypes and strains of *C. botulinum* and *C. tetani* (Boroff and DasGupta, 1971; Schantz and Johnson, 1992).

Ideally, studies of metabolic and genetic regulation should primarily use well-characterized strains since different strains vary in metabolic patterns, toxin production and regulation, and genomic structure. Generally, investigations of TeNT production have used the Harvard strain developed by Mueller and colleagues (Mueller and Miller, 1954), which has allowed for direct comparisons of results among laboratories. In contrast, many different strains of *C. botulinum* have been used in studies of toxin production, even within a single serotype.

Our laboratory has investigated molecular aspects of the regulation of toxigenesis by a variety of approaches. Recently we have determined the kinetics of transcription of toxin complex genes and have quantitated the production of intracellular and extracellular BoNT/A during the growth cycle. This analysis was performed using three strains (Hall A, 62 A, and NCTC 2916) in two media (TPGY and toxin production medium, TPM). The three strains differed markedly in their patterns of growth, lysis and toxin formation, and post-translational nicking. These patterns depended on the growth medium. A manuscript is in preparation and the results will not be discussed in detail in this review. However, it is interesting to note that the differences observed in patterns of growth and toxin production indicate that the strains may have fundamental differences in their regulatory mechanisms of toxigenesis.

For large scale production, TeNT and BoNT are generally produced in fermentors, while for smaller lots static flasks or carboys are adequate to obtain several hundred micrograms or milligrams of toxin. Siegel and Metzger (1979, 1980) reported conditions in fermentors to achieve reasonable toxin production by the Hall A and Bean B strains of *C. botulinum*. Toxin can also be produced in increased concentrations in dialysis membranes immersed within the bulk culture medium, in which the cells and protein toxins cannot diffuse through the membrane (Wentzel and Sterne, 1949). Although not reported for *C. botulinum* or *C. tetani*, it has been found that cell density and cell-to-cell signalling affects toxin production in many gram-positive pathogens including *Staphylococcus aureus* (Kleerebezem et al., 1997; Ji et al., 1995) and *C. difficile* (Roberts et al., 2000). Cell-to-cell signalling would be expected to be a beneficial mechanism for regulating the acquisition of nutrients, as well as for toxin production and intoxication of prey, since a mimimum cell density would be necessary to promote growth of the clostridial population without excessively benefiting other bacterial species in the environment. It would be useful to conduct careful studies of TeNT and BoNT production in chemostats where cell density and limiting nutrient concentration can be precisely controlled.

TeNT production and BoNT production in type A and B *C. botulinum* is markedly influenced by nitrogen and carbon nutrition (Leyer and Johnson, 1990; Patterson-Curtis and Johnson, 1989; Schantz and Johnson, 1992). The production and assembly of toxin complexes is also influenced by the presence of metals in the growth medium (Lamanna et al., 1946; Schantz and Johnson, 1992; Wentzel and Sterne, 1946). The production of TeNT was affected by the availability of histidine, and histidine-containing peptides induced synthesis while free histidine repressed its formation (Mueller and Miller, 1956). Recently, Porfirio et al. (1997) showed that peptides that contain one or two residues of proline and a predominance of hydrophobic amino acids stimulated TeNT production, and the most active peptides had the general structure of proline-aromatic-proline, resembling the motifs of bradykinin-potentiating peptides found in snake venoms. They suggested that these peptides in the medium could protect secreted TeNT from proteolysis, in an analogous manner to the potentiation of bradykinin by inhibition of proteolysis. Consistent production of TeNT and BoNTs with high yields and good quality requires that the medium components be carefully screened for production, since even different lots of the 'same' casein digests and other nutrients affect toxin production and quality. For vaccine or pharmaceutical use, BoNT and TeNT are still primarily produced in complex media, although it has long been recognized that a defined medium is preferred for production since it would be possible 'to obtain a uniform product free from any possible antigenic material other than the specific substance desired' (Mueller and Miller, 1942). The value of using chemically defined medium components would also prevent a product from being administered to humans that may contain prions, pathogenic viruses, or other bioburdens.

In complex media it is often difficult to accurately assess the effects of specific nutrients on the expression of toxin or the pathways of nutrient utilization affecting toxin synthesis (Barker, 1981). Chemically-defined minimal media were developed to study the physiology of *C. botulinum* types A, B, and E (Whitmer and Johnson, 1988), and these have proved useful for studies of toxin regulation (Patterson-Curtis and Johnson, 1989; Leyer and Johnson, 1990). Chemically-defined medium have also been beneficial for study of toxin regulation in *C. tetani* (Mueller and Miller, 1956; Porfirio et al., 1997). Supplementation of chemically-defined minimal medium with high levels of arginine (20 g/l) markedly decreased BoNT titers and protease activities in *C. botulinum* Hall A and Okra B (Patterson-Curtis and Johnson, 1989). Nitrogenous nutrients that are known to be derived from arginine, including proline, glutamate, and ammonia, also decreased protease and toxin but less so

than did arginine. Similarly, nitrogenous nutrients, particularly the aromatic amino acid tryptophan, markedly decreased toxin production in *C. botulinum* serotype E (Leyer and Johnson, 1990). Growth and toxin production in strains of *C. difficile* has also been reported to depend on nutrients and growth conditions, particularly the amino acid composition of the medium (Haslam et al., 1986), including arginine (Karasawa et al., 1997), and also biotin (Yamakawa et al., 1996) and glucose (Dupuy and Sonenshein, 1998). Regulation of synthesis of clostridial toxins nitrogenous nutrients and by glucose suggests that a primary controlling mechanism is nitrogen regulation and possibly catabolite repression. These important and general regulatory mechanisms have not been characterized in clostridia. In enteric bacteria, genes expressed under nitrogen limitation are controlled by a cascade of regulatory proteins that activate promoters controlled by different sigma factors (Zimmer et al., 2001). Among the many operons activated by NtrC/Nac under nitrogen limitation include those involved in arginine catabolism and a number of transport operons. An analogous NTR system has not been shown in *Clostridium* or the well-characterized spore-former *Bacillus subtilis* (Fisher and Sonenshein, 1991). Genes coding for alternative sigma factors have been found in *Clostridium* and have been postulated to regulate spore formation (Arcuri et al., 2000; Santangelo et al., 1998; Wösten, 1998), but the research is in early stages. In *B. subtilis*, catabolite repression involves a *cre* sequence coding for CRE regulatory protein (catabolite repression element) (Hueck and Hillen, 1995), but *C. difficile* strains examined did not have detectable *cre* sequences (Dupuy and Sonenshein, 1998) and thus catabolite repression in certain clostridia probably involves different mechanisms.

The mechanism of release or secretion of BoNT and TeNT are not known. TeNT and BoNT from *C. botulinum* strain 62A were mainly located intracellularly or attached to the bacterial cell throughout growth (Call et al., 1995; Habermann and Dreyer, 1986), whereas in some strains (particularly Hall) BoNT appears to be released into the culture fluid by cell autolysis late in the growth cycle (Schantz and Johnson, 1992). The onset and extent of autolysis varies with serotype, strain, and medium components. Post-translational modifications of TeNT and BoNT such as nicking were reported to occur intracellularly in certain strains of *C. tetani* and *C. botulinum* (Habermann and Dreyer, 1986). However, single chain BoNT of ~150 kDa was detected intracellularly in *C. botulinum* during exponential growth (Krysinski and Sugiyama, 1981). It appears likely that nicking occurs during secretion or extracellularly in the culture fluid. Nicking and oxidative linkage of the disulfide linking the H and L chains would occur preferentially during secretion or extracellularly, since the cellular cytoplasm has a reducing environment. Disulfide bond formation in proteins has been studied extensively in *E. coli* by Beckwith and colleagues. Except in mutants that have altered control of intracellular redox activity, disulfide bond formation in proteins takes place in the periplasmic space in an oxidizing environment (Debarbieux and Beckwith, 1999). BoNT is also subject to certain extracellular modifications on extended incubation, particularly nicking and fragmentation with losses in specific toxicity. In order to optimize BoNT quality, it will be valuable to accurately determine the kinetics of synthesis of the proteins that form the toxin complexes, and to elucidate factors influencing their stability and assembly into the complexes.

7. Structure and organization of BoNT complexes

Although the neurotoxigenic clostridia are physiologically and phylogenetically diverse, there is uniformity in the organization of the genes encoding the toxin complexes (also termed progenitor toxins). The association of BoNT with other proteins was initially shown by ultracentrifugation in alkaline conditions (Wagman and Bateman, 1951) and by the demonstration that the neurotoxic organisms and hemaglutinin activity could be dissociated (Lamanna and Lowenthal, 1951). Sakaguchi and colleagues purified and characterized all seven toxin complexes (Oguma et al., 2000; Sakaguchi et al., 1988). The toxin complexes that are transcribed, translated and secreted/assembled vary in structure among the various serotypes and strains. The toxin complexes range in size from ~300 to 900 kDa depending on the serotype, strain, nutritional and environmental conditions of culture, and method of isolation (Schantz and Johnson, 1992). Various nutritional and cultural factors including metal composition of the culture medium, presence of amino acids and peptides, pH and buffering capacity, temperature, and cell density have been reported to influence toxin complex production and structure (Schantz and Johnson, 1992).

Other than the BoNT component of the complex which has catalytic activity on neuronal substrates (Schiavo et al., 2000), very little is known of the roles of the proteins and nucleic acid components of these complexes. The nontoxic proteins of the complexes are often discarded during purification of the BoNTs, and their structures and possible functions have not been studied in detail. Evidence indicates that some of the nontoxic proteins have protective roles, and help to retain BoNT activity during oral ingestion (Oguma et al., 2000; Sakaguchi et al., 1988), and on high dilution which is necessary for compounding as a pharmaceutical (Schantz and Johnson, 1992, 1997). The 'adjuvant' proteins also may help slow diffusion of BoNT into neighboring tissues and resulting ptosis on injection as a pharmaceutical. BoNT dissociates from the nontoxic proteins in the lymph and in the blood. Some of the HA components have amino acid sequence homology with the region of *C. perfringens* enterotoxin that is important for insertion into target cells, and they also possess Arg-Gly-Asp (RGD) and Lys-Gly-Asp (KGD) sequences similar to regions of fibronectin, and thus may promote interaction with integrin, the cell surface receptor for fibronectin (Oguma et al., 2000). Balding et

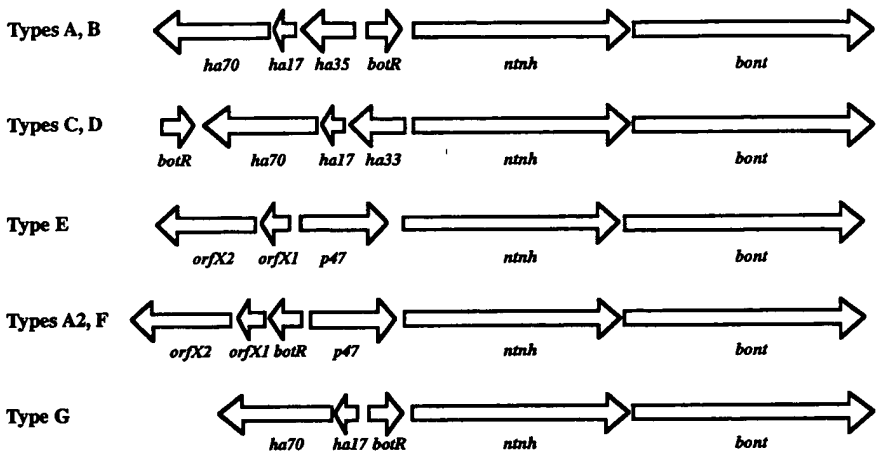

Fig. 1. Arrangement of the genes in the neurotoxin gene clusters of *C. botulinum*.

al. (1973) also demonstrated that HA preparations from *C. botulinum* types A and D bound weakly when target tissue was treated with neuramidase and that binding was inhibited by Pneumococcus capsule containing D-galactose as its terminal sugar. Later, it was shown that BoNT complex had affinity for galactose residues (DasGupta and Sugiyama, 1977), and that gangliosides inhibited the hemagglutination reaction (Suzuki et al., 1986). Thus, it appears that HA may bind to receptors on cells that contain sialic acid and/or D-galactose on the surface, but the receptors have not been identified.

All of the BoNT genes analyzed to date reside in clusters together with the gene immediately upstream that codes for NTNH (Fig. 1). In many BoNT complexes, a four-component HA is also present, which is encoded by three genes in one operon. Most clusters also have a positive regulatory gene, *botR*, encoding a protein of about 21 kDa (Popoff and Marvaud, 1999), and some clusters have additional uncharacterized ORFs (Kubota et al., 1998). Certain strains of *C.botulinum* type A associated with infant botulism lack the HA genes (Kubota et al., 1996; Tabita et al., 1991), and certain strains contain silent genes for NTNH and BoNT that are not expressed due to various classes of mutations (Hutson et al., 1996). It was suggested (Kubota et al., 1998) that the gene arrangement of the botulinum toxin complexes could be divided into three groups based on the gene organization of *ha, p21[botR], p47*: ClassI (*C. botulinum* types A and B; *-ha-p21[botR]-ntnh-bont-*); class II (*C. botulinum* types C and D; *-p21[botR]-ha-ntnh-bont-*); and class III (*C. botulinum* type F and *C. botulinum* type A isolated from an infant botulism case in Japan (Kyoto F strain); *-p21[botR]-p47-ntnh-bont-*). Type G was not included in the classification. In this scheme, the organization of the genes encoding NTNH and BoNT is the same, while those of *ha, p21[botR]*, and *p47* are different among *C. botulinum* serotypes characterized (Kubota et al., 1998). Further characterization of toxigenic *C. butyricum* showed the presence of additional ORFs (encoded by ORF-X1 and ORF-X2) in the type E, F, Infant A, and toxigenic *C. butyricum* strains analyzed, and had variable distribution of *orf22* or *p21 [botR]*, and *p47* (Kubota et al., 1998). The genes and their sequences have been extensively analyzed for regions of homology and phylogenetic relationships (Collins and East, 1998; Henderson et al., 1997; Lacy and Stevens, 1999; Minton, 1995; Popoff and Marvaud, 1999).

The genes encoding the BoNT complexes are assumed to be 'chromosomally located' in *C. botulinum* serotypes A, B, E, and F, and are present on pseudolysogenic bacteriophages in types C and D, and on large plasmids for the BoNT/G complex (Eklund et al., 1989; Minton, 1995; Henderson et al., 1997; Zhou et al., 1995). In the absence of evidence showing involvement of phages, plasmids, or other moveable or extrachromosomal elements the designation of A, B, E, and F as being 'chromosomally located' is assumed. In our work in *C. botulinum* strain 62A, Tn*916* mutagenesis, PFGE and DNA hybridization analyses clearly proved that the toxin gene cluster is chromosomally located (Johnson et al., 1997; Lin and Johnson, 1991, 1995). It is surprising that more genes have not been mapped by this approach using PFGE and DNA hybridization in *C. botulinum* compared to certain other clostridia (Lyras and Rood, 2000). The variation in the complement and organization of genes coding for the complexes as well as the detection of silent or chimeric (see below) toxin gene complexes in numerous strains indicates that considerable gene movement and possibly transposition and/or recombination occurs within these regions in *C. botulinum* and toxigenic *C. butyricum*. PFGE analysis indicated that the silent and active toxin gene clusters were separated by 40–60 kb on the chromosome in the two strains examined (Hutson et al., 1996). Interestingly, sequence analysis of NTNH adjacent to the defective BoNT/B gene was 'chimeric', the 5′- and 3′ regions of the gene showing high homology with the corresponding regions of the B NTNH gene, while the 471-amino acid

sequence in the central region was identical to NTNH of type A (Hutson et al., 1996). A 'mosaic' toxin gene was also detected in three strains of *C. botulinum* that consisted of parts of BoNT/C1 and BoNT/D (Moriishi et al., 1996). The chromosomal regions flanking complex have not been well characterized and the genes are not known, other than a putative autolysin gene located downstream from the toxin gene cluster in *C. botulinum* NCTC 2916 (Minton, 1995). Similar sequences were found in only a few other strains (Minton, 1995). Unpublished data from our laboratory indicate that there is considerable variation in the upstream and downstream regions in three type A *C. botulinum* strains being characterized. Variations in these regions could explain differences in toxin production observed in different type A strains.

8. Positive and negative regulation of BoNT expression

The positive regulatory proteins, BotR and TetR, have characteristics of transcriptional regulators including basic pIs (10.4 and 9.3, respectively) and helix-turn-helix motif structures. BotR and TetR possess considerable homology (~50% identity) have 20–29% identity to some other regulatory proteins including UviA, a putative activator of bacteriocin synthesis in *C. perfringens*, and TxeR from *C. difficile*, a positive activator of toxins A and B (Marvaud et al., 1998a,b). Studies have been performed to determine the regulatory mechanisms by which BotR/A and TetR activate transcription. BotR/A was demonstrated to bind to promoter regions of the *ntnh* and *ha35* genes, and impeded gel mobility of the gene regions, supporting that BotR/A activated both operons (Marvaud et al., 1998b). TetR and BotR/A were overexpressed in their respective hosts from high copy number plasmids containing *tetR* or *botR/A* genes under control of their own promoters. The vectors were transferred to *C. botulinum* and *C. tetani* by electroporation. The presence of extrachromosomally synthesized tetR or botR/A resulted in moderately increased production (5–12-fold) of TeNT or BoNT and other nontoxic components in the BoNT toxin complex. When antisense RNA was introduced, lower production of TeNT and BoNT and nontoxic components were observed, supporting that *tetR* and *botR/A* are positive regulatory genes. Interestingly, BotR/A and BotR/C were also functional in *C. tetani* (Popoff and Marvaud, 1999).

Positive regulation by TxeR has also been investigated in *C. difficile* and it is of interest to compare the results to those obtained with botR/A and tetR in *C. botulinum* type A and in *C. tetani*. In contrast to the modest increase in expression of toxin genes observed in *C. botulinum* and *C. tetani* by extrachromosomally synthesized BotR/A and TetR, the 22 kDa positive regulator txeR from *C. difficile* increased expression of transcriptional fusions in *E. coli* over 500-fold from the toxin A promoter and over 800-fold from the toxin B promoter (Moncrief et al., 1997). Due to difficulties in gene transfer in *C. difficile*, it was not possible to study regulation of toxin gene expression in the native organism. However, the much higher positive expression in *C. difficile* compared to *C. botulinum* suggests that expression in *E. coli* avoided the influence of negative regulators (Hundsberger et al., 1997) present in the native host. In concordance with expression of virulence genes in other organisms (Strauss and Falkow, 1997), BoNT and TeNT formation is probably controlled by two component systems involving positive and negative regulation.

Our laboratory has found evidence for the involvement of negative regulatory factor(s) in BoNT production (Bradshaw et al., 1998). Negative regulation was supported by expression studies carried out in *C. botulinum* 62A and its nontoxigenic derivative LNT01–transposon Tn*916* mutant strain lacking the entire toxin gene cluster (Johnson et al., 1997; Lin and Johnson, 1991). A hybrid gene was constructed that contained a recombinant light chain (LC) derivative of BoNT/A (pMVP108) (Fig. 2A) which differed in size from the native light chain. The hybrid BoNT LC was constructed so as to preserve the structural and putative regulatory components in the toxin gene cluster: the region coding for the *BotR/A* gene and it's promoter, the *ntnh* promoter and the region coding for the first 69 amino acid residues of the *ntnh* gene were fused in frame with the *bont* LC (Fig. 2A) (Bradshaw et al., 1998). This construct provided a translational fusion that allowed monitoring of the expression of BoNT light chain sequences. The construct was transferred from *E. coli* S17-1 to *C. botulinum* strains 62A and LNT01 using the shuttle plasmid pMVP108 (Bradshaw et al., 1998), and selected transconjugants were tested for expression of the NTNH-LC gene fusion on Western blots. Interestingly, LNT01 containing the fusion construct pMVP108 produced considerably more recombinant LC than did 62A with the construct (Fig. 2B). These results suggested that negative regulatory factors were present in 62A but were absent in LNT01. A second construct (pMVP129) was prepared that did not contain *BotR/A* in front of the *ntnh* promoter (Fig. 2A). Strikingly, no expression of recombinant BoNT LC was observed in LNT01, while small amounts were expressed in *C. botulinum* 62A (Fig. 2B). The quantities of recombinant BoNT LC expressed from pMVP129 were less than those from pMVP108 in *C. botulinum* 62A. These results showed that BotR/A is a positive regulator, and is essential for BoNT expression. The smaller quantity of recombinant BoNT LC produced in 62A from pMVP129 was due to the BotR/A provided by the chromosomal copy of *BotR/A*. Furthermore, the results suggested that additional factors are involved in regulation of BotR/A expression, whereby a cascade controls expression of genes for the toxin complex. These factors apparently are not present in strain LNT01, because increased expression of the hybrid gene was observed in this strain. Since strain LNT01 lacks the entire toxin gene cluster and its flanking regions, it appears reasonable that sequences located near or in some distance to the cluster are involved

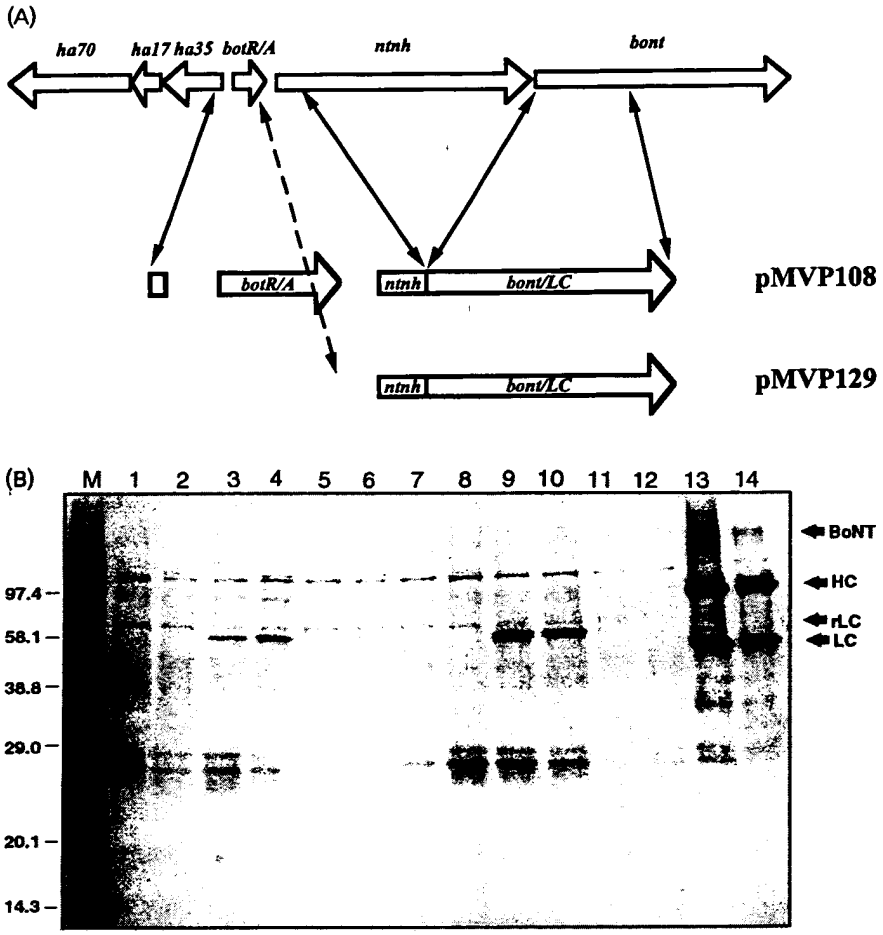

Fig. 2. Structure and expression of recombinant LC gene constructs in *C. botulinum* strains 62A and LNT01. (A) Schematic representation of the hybrid gene constructs pMVP108 and pMVP129. The arrangement of the genes in the botulinal toxin gene cluster is shown on top, and the lower portion shows the botulinum regions used to construct the hybrid NTNH-LC gene. (B) Native and recombinant proteins were separated by SDS–PAGE and transferred to a PVDF membrane. The Western immunoblot was reacted with polyclonal antibodies specific to *C. botulinum* type A neurotoxin as described in Bradshaw et al. (1998). Lane M: molecular weight markers in kDa; lane 1: 62A; lane 2: 62A (pJIR1457); lanes 3 and 4: 62A (pMVP108); lanes 5 and 6: 62A (pMVP129); lane7: LNT01; lane 8: LNT01(pJIR1457); lanes 9 and 10: LNT01(pMVP108); lanes 11 and 12: LNT01(pMVP129); lanes 13 and 14: BoNT. Arrows at the right side of the Western blot indicate the positions of the native light chain (LC), recombinant light chain (rLC), heavy chain (HC) and BoNT.

in regulation of toxin cluster genes by controlling synthesis of BotR/A. These experiments were also the first demonstration of transfer and expression of cloned botulinal genes in *C. botulinum*. Further experiments are being performed to identify genes regulating BoNT synthesis.

9. Genetic approaches for study of *C. botulinum* and expression of BoNT

9.1. Overview

Although gene manipulation methods have been established in *C. perfringens* (Lyras and Rood, 2000; Rood, 1998) and in *C. acetobutylicum* (Mitchell, 1998), genetic methods have developed comparatively slowly in *C. botulinum*. Genetic analysis of toxinogenesis and other properties in *C. botulinum* has been hindered because of the lack of certain needed tools, including directed mutation systems, efficient gene transfer methods, lack of cloning and expression vectors, and absence of gene replacement strategies (Minton, 1995; Lyras and Rood, 2000; Young et al., 1989). Certain genetic tools are in various stages of development in *C. botulinum* including improved cloning, shuttle, mutagenesis, and expression vectors (Bradshaw et al., 1998; Davis et al., 2000; Dineen et al., 2000; Marvaud et al., 1998b), the use of transposons for mutagenesis and gene manipulation (Lin and Johnson, 1991), transformation

systems (Bradshaw et al., 1998; Davis et al., 2000; Marvaud et al., 1998b; Zhou & Johnson, 1993; Zhou et al., 1993), and reporter systems (Davis et al., 2000; Schmidt, J., 1998. M. S. Thesis, University of Wisconsin, Madison). In the following sections, gene manipulation methods in *C. botulinum* are briefly reviewed.

9.2. Chemical mutagenesis of clostridia

The isolation of mutants in clostridia and certain other anaerobes by spontaneous mutation, chemical mutagenesis or by physical techniques such as UV irradiation varies in efficacy depending on the treatment used and the target organism. Methods used for obtaining mutants, possible mutagenesis pathways, and a compilation of mutants isolated has been reviewed by Sebald (1993b). Generally, isolation of mutants in anaerobes by chemical or physical mutagenesis has been more difficult than with aerobes. Since methods of genetic analysis are lacking in most anaerobes, mutants obtained by chemical or physical mutagenesis are usually difficult to map and characterize. For this reason, directed mutagenesis approaches such as using conjugative plasmid or transposon systems have been of greater utility in the clostridia.

9.3. Transposon mutagenesis and manipulations in clostridia

Transposons, particularly conjugative and promiscuous transposable elements, have been useful in gene manipulations of bacterial species in which genetic systems are poorly established (Clewell and Flannagan, 1993; Wong and Mekalanos, 2000), including many clostridial species (Lyras and Rood, 1997; Mitchell, 1998; Young, 1993). Transposons and IS elements have not been reported to occur naturally in *C. botulinum*, but they have been found in other pathogenic clostridia including *C. difficile* and *C. perfringens* (Lyras and Rood, 1997, 2000).

Our laboratory has used Tn*916*, the well-characterized 18 kb tetracyline resistant conjugative tranposon (Flannagan et al., 1994) to isolate mutants affected in various phenotypic properties (Lin and Johnson, 1991). Although the transfer frequency of Tn*916* from *E. faecalis* to *C. botulinum* 62A and 113B is somewhat low (about 10^{-4}–10^{-7} tetracycline-resistant transconjugants per recipient depending on the donor strain), this frequency is of sufficient magnitude to generate a relatively large pool of transconjugants for screening or selection. Recently, by performing all manipulations under strictly anaerobic conditions, the Tn*916* conjugation process has been improved and we easily obtain large numbers of transconjugants. Tn*916* stably integrates into random regions of the *C. botulinum* chromosome as shown by PFGE and DNA hybridizations (Lin and Johnson, 1991, 1995). The random integration and apparent lack of hot spots of Tn*916* integration differs from *C. difficile* (Wang et al., 2000) and *C. perfringens* (Awad and Rood, 1997), in which Tn*916* preferentially integrates into specific loci ('hot spots'). Integration of Tn*916* into *C. botulinum* type A and B chromosomes resulted in auxotrophic mutations and other classes of mutants. To isolate mutants affected in toxinogenesis, randomly isolated tetracycline-resistant transconjugants were screened for toxin surrounding the colonies by a colony immunoblot assay using affinity purified antibodies (Goodnough et al., 1993). The immunoblot assay can also distinguish colonies that show increased or decreased toxin production. Since there is no known cellular function for BoNT in *C. botulinum*, the screening of individual colonies for toxin production is very labor intensive. Of an estimated 7000 colonies screened in early study (Lin and Johnson, 1991), we isolated three mutants affected in toxigenesis. Interestingly, two mutants (LNT01 and LNT03) had a large deletion of ca. 70 kb as determined by PFGE and DNA hybridization of the restriction fragment containing the toxin gene cluster. In these mutants, the entire toxin gene cluster was deleted. The mechanism by which Tn*916* caused the large deletion has not been elucidated. LNT01 also had other unexpected properties including hypersporulation, and inability to grow in minimal medium. The genotypic properties of the three mutants were characterized by a variety of methods (Johnson et al., 1997). The data with three independent mutants indicated that the toxin gene cluster of *C. botulinum* 62A lies within a region that can be lost from the genome by a deletion event, suggesting that a moveable element capable of carrying a large piece of DNA could be involved in toxin gene mobilization. The most likely candidate is a prophage that could carry the genes of the toxin complex, but efforts to isolate this genetic element so far have been unsuccessful. In recent work, we have isolated several Tn*916* transconjugants that are altered in toxin formation. These mutants are being analyzed in detail to determine if there are additional genes that control toxin production.

9.4. Bacteriophages and transduction in toxigenic clostridia

Bacteriophages are well known to be present in a number of *Clostridium* species (Eklund, 1993; Eklund et al., 1989; Johnson, 1997). Phages have been conclusively demonstrated to carry toxin genes in *C. botulinum* types C and D, and to facilitate interspecies transfer of C1 (neurotoxin) and C2 (binary toxin) genes between *C. botulinum* C and D serotypes and *C. novyi* (Eklund, 1993; Eklund et al., 1989). In groups I and II of *C. botulinum*, however, bacteriophages have not been demonstrated to harbor toxin genes nor has successful transduction been achieved (Eklund, 1993). The inability to obtain transduction is due mainly to obtain indicatory strains sensitive to infection that form plaques in agar plates. Thus, until methods for infection and phage sensitivity can be achieved, transduction does not appear to be a feasible method for genetic manipulation in *C. botulinum*.

9.5. Transformation of plasmids into C. botulinum by electroporation

Transformation using electroporation has been limited to only a few strains of group I (proteolytic) *C. botulinum* (Marvaud et al., 1998b; Zhou et al., 1993) and the frequency of transformants has been low (~10^2–10^3 transformants per μg DNA). Recently, a transformation system was reported for Group II (nonproteolytic) *C. botulinum* type B strains with higher frequencies of up to 0.8×10^4 transformants per μg of DNA (Davis et al., 2000). To obtain this frequency, it was necessary to bypass endonuclease and methylase activities constituting restriction barriers in *C. botulinum* type B. However, in our experience, introduction of plasmids by transformation is more labor intensive, requires specific equipment and additional treatments for plasmid preparation, and is significantly less efficient than conjugal transfer (Bradshaw et al., 1998; Lin and Johnson, 1991).

9.6. Shuttle and expression vector systems for gene transfer, manipulation and gene expression in C. botulinum

Although *C. botulinum* harbors many plasmids (Eklund, 1993; Eklund et al., 1989; Johnson, 1997; Young, 1993), most have cryptic functions, and conjugative plasmids carrying antibiotic resistance markers have not been reported. Plasmids in *C. botulinum* have been shown to harbor genes for BoNTs (Hauser et al., 1995; Zhou et al., 1995), and a bacteriocin (Dineen et al., 2000), however, the plasmids have not been analyzed in detail. Furthermore, most of *C. botulinum* plasmids replicate via the rolling circle mechanism, generating single-stranded intermediate molecules, known to stimulate illegitimate recombination, thus potentially causing structural instability of the plasmids. Due to these properties of known native *C. botulinum* plasmids they have not been used to develop vectors.

Gene transfer systems have been developed for *C. perfringens* and *C. acetobutylicum* (Lyras and Rood, 2000; Rood, 1993; Minton et al., 1993), which has facilitated intensive genetic analyses of these two clostridial species. Rood and colleagues have constructed numerous vectors using *C. perfringens* well-characterized endogenous plasmid components, including elements of replication and antibiotic resistance markers (Lyras and Rood, 1998, 2000; Rood, 1993). On the other hand, many of the vectors used in studies of *C. acetobutylicum* are composed of heterologous plasmid components (Minton et al., 1993). All these vectors contain a multicloning region, *E. coli* replication origin, antibiotic resistance marker functional in *E. coli*, and some carry a β-galactosidase α-peptide, sequences necessary for plasmid maintenance in *E. coli*, and sequences facilitating insertion of the cloned genes. To date, three different vectors have been used to transfer cloned DNA sequences to *C. botulinum*.

We have adapted one of the *E. coli– C. perfringens* shuttle vectors, pJIR1457 (Lyras and Rood, 1998) for transfer of cloned genes into *C. botulinum* (Bradshaw et al., 1998). This vector contains the replication origin *oriCP* and replication gene *rep* from *C. perfringens* plasmid pIP404, *C. perfringens* erythromycin resistance gene (*ermBP*); it also carries an origin of conjugative transfer, RP4 *oriT*. The vector was developed for mobilization into *C. perfringens* from a suitable *E. coli* donor.

In our studies, shuttle vector pJIR1457 was initially transferred by conjugation from *E. coli* to *C. botulinum* type A strains and to nontoxigenic LNT01. Randomly selected erythromycin resistant colonies were analyzed and shown to contain unaltered pJIR1457 (Bradshaw et al., 1998), indicating that *C. perfringens* replication signals and ermBP are functional in *C. botulinum*. The frequency of transfer depended on the recipient strain but ranged from 10^{-3} to 10^{-4} recipients per donor cell, which is similar to the frequency for transfer of the plasmid to *C. perfringens* (Lyras and Rood, 1998), and is higher than the frequency of conjugative transfer of Tn*916* from *Enterococcus faecalis* to *C. botulinum* (Lin and Johnson, 1991). Conjugal transfer was also about two logs higher than transformation achieved by electroporation (Zhou et al., 1993), and was also not limited to the Hall A strain as the transformation recipient.

Marvaud et al. (1998a,b) have used a high-copy number shuttle vector pAT19 (Trieu-Cuot et al., 1991) to overexpress *botR/A* and *tetR* genes in *C. botulinum* and *C. tetani*. The vector contains the replication origin and replication gene *orfE* from the broad host range enterococcal plasmid pAMβ1, the erythromycin resistance gene from the conjugative transposon Tn*1545* from *S. pneumoniae*, and an origin of conjugal transfer of the *Inc*P plasmid RK2. Despite having conjugation properties, the authors used electroporation to transfer *botR* and *tetR* expression plasmids to *C. botulinum* and *C. tetani*.

Davis et al. (2000) have used the previously described clostridial cloning and expression vectors pMTL500E and pMTL540E (Minton et al., 1993). These vectors also contain the replication origin and replication gene(s) from the plasmid pAMβ1, and the erythromycin gene from the same plasmid. This plasmid is designed to optimize expression of native or reporter genes (Davis et al., 2000). This plasmid has been introduced into group II *C. botulinum* type B by electroporation.

In general, at least two different replication origins are shown to be functional in *C. botulinum*, and the plasmids appear to be reasonably stable and replicate by the theta mechanism thereby preventing formation of unstable single stranded derivatives. The efficiency of plasmid introduction into *C. botulinum* depends on the transfer technique selected, followed by appropriate measures to protect the plasmid DNA from *C. botulinum* restriction and modification systems. Further studies are needed to evaluate the interaction of introduced plasmids with native cryptic plasmids, which can lead to instability or other undesired recombination events. Nonetheless, the results obtained so far are

promising for advanced gene manipulation systems in *C. botulinum*.

9.7. Homologous recombination and gene replacement systems in C. botulinum

As described by Rood (1997), reverse genetics and gene inactivation or replacment by homologous recombination is a very useful method for analyzing gene function in bacteria. Successful methods have been developed in a number of gram-positive organisms (e.g. see reviews for other organisms: Carparon, 2000; Duwat et al., 2000; Firth and Skurray, 2000; Freitag, 2000). Using homologous gene replacement, it becomes possible to specifically test the functions of individual structural or regulatory genes in complex bacterial phenotypes such as toxin production. Often suicide vectors are introduced that are unable to replicate, or are conditional (e.g. temperature sensitive) for replication, and the only way in which the plasmids or constituent genes can survive are by integration into the chromosome (Rood, 1997). Allelic exchange has been used to construct defined mutants in *C. perfringens* and to a more limited degree in *C. difficile* (Lyras and Rood, 2000; Mullany et al., 1994), but not in other toxigenic clostridia.

9.8. Reporter systems for C. botulinum

Gene fusions and reporter systems have been immensely successful in studying expression and function of genes in various organisms (Thorner et al., 2000). Reporter systems using in-frame transcriptional fusions with various gene promoters have been constructed for *C. perfringens* (Bullifent et al., 1995; Phillips-Jones, 2000), *C. beijerinckii* (Quixley and Reid, 2000), *C. acetobutylicum* (Tummala et al., 1999), Group II (nonproteolytic) type B *C. botulinum* (Davis et al., 2000), and *C. botulinum* type A (Schmidt, 1998; Schmidt et al., unpublished data). Potentially, the use of transcriptional and translational fusions could provide excellent systems for studying regulation of gene expression and production of chimeric and hybrid proteins in the clostridia, and for evaluating in vivo pathogenesis. However, several reporter genes such as luciferase and green fluorescent protein require molecular oxygen for assay (Lux) or correct folding of the protein (GFP), and thus there are limitations in the use of these reporter proteins with anaerobic clostridia. Furthermore, we found that the gene encoding firefly luciferase was poorly expressed in *C. botulinum* type A compared to in *E. coli* (Schmidt, 1998. M.S. Thesis, University of Wisconsin–Madison).

Recently, a reporter system was developed for nonproteolytic group II *C. botulinum* type B (Davis et al., 2000). The *lacZ* gene from *Thermanaerobacterium sulfigigenes* and the *luxAB* genes from *Vibrio fishceri* were evaluated as reporters. LacZ was better expressed than LuxAB from a transcriptional fusion to the promoter region of the BoNT gene. However, toxin production was not determined simultaneously with reporter expression, and thus the correlation between reporter activity and toxin synthesis remains to be determined. Although the plasmid containing the reporter system was quite stable during repeated subculture, attempts to integrate the reporter cassette into the chromosome for maximum stability were unsuccessful. It was suggested that this reporter system could be used to evaluate toxin gene expression without using the mouse bioassay in foods and other environments, but more research is necessary to validate this hypothesis.

9.9. Stability of gene expression in clostridia

Toxin formation as well as other clostridial processes such as solvent formation are generally unstable traits and can be lost after repeated subculture (degeneration) (Bahl et al., 1982; Kashket and Cao, 1995). Spontaneous degenerate mutants readily occur in laboratory culture. In clostridia for which degenerate genes have been studied, unstable genes often occur on large plasmids (Cornillot and Soucaille, 1996) or on bacteriophages (*C. botulinum* types C and D; Eklund et al., 1989). In *C. acetobutylicum*, loss of the pWEIZ plasmid encoding solvent-forming genes also resulted in that did not sporulate (Cornillot and Soucaille, 1996), suggesting involvement of common regulatory elements in loss of the traits. It has been suggested that a regulatory region and perhaps a specific regulatory gene is involved in strain degeneration in *C. acetobutylicum* (Kashket and Cao, 1995) In *C. botulinum* type A, a strain that lost the neurotoxin gene cluster had a hypersporulation phenotype (Lin and Johnson, 1991). It has also been reported that toxigenic cells of *C. tetani* did not contain spores (Habermann and Dreyer, 1986). Thus, common regulatory genes may be involved in toxigenesis and sporulation.

9.10. Recombinant toxin production

Due to the technical difficulties in studying strict anaerobes together with the lack of genetic tools, several groups have developed methods to express clostridial toxins and associated proteins in heterologous hosts. The fidelity of expression, yields, and biological activities of the proteins expressed in heterologous hosts depend on several factors including the heterologous host, the expression vector and its genetic determinants, the nature of the gene or gene fragment that is expressed, and the metabolic and genetic deregulation of synthesis. Many clostridial genes have extremely low G + C content, with certain codons having a strong bias for preferred usage of A or U in the degenerate position (Rabinowitz, 1993; Young et al., 1989). However, codon bias and resulting rate of polypeptide elongation is only one of the impediments to clostridial gene expression in heterologous hosts, synthesis is also influenced by other characteristics of the heterologous gene including promoter structure, recognition by RNA polymerase and efficiency of transcription, mRNA stability during and

following transcription, translation efficiency, solubility, and folding and stability of the foreign protein (De Voss et al., 1997; Graves and Rabinowitz, 1986; Kurland, 1991; Rabinowitz, 1993). Strategies to overcome these impediments have been used to increase expression, while for optimal expression a number of manipulations may be necessary (Kurland, 1991).

Fragments of BoNT and TeNT genes have been expressed in heterologous systems, although yields have varied considerably depending on the strategy used for expression and the characteristics of the heterologous gene(s). The pioneering research involved the expression of tetanus toxin fragments in *E. coli* (Eisel et al., 1986; Fairweather and Lyness, 1986; Fairweather et al., 1986). Tetanus toxin fragment C was initially expressed at low levels and was insoluble in the cytoplasm of *E. coli*, but was later expressed in higher quantities and in a soluble form using a high expressing plasmid and altered N-terminal region including a methionine (Makoff et al., 1989a). Even higher expression of fragment C was achieved in *E. coli* by removing rare codons seldomly used in this host (Makoff et al., 1989b). In a similar approach, synthetic gene sequences were constructed to eliminate rare codons from the Hc fragments of BoNTs (Clayton et al., 1995), and the protein fragments have produced in *E. coli* and the yeast *Pichia pastoris* (Byrne et al., 1998, 2000; Smith, 1998). Although synthetic genes compatible with the heterologous host have yielded high quantities of protein in the yeast system, glycosylation and a slightly different tertiary structure (Byrne et al., 2000) for some serotypes may preclude certain uses of the products. A synthetic gene for expression of the Hc fragment of BoNT/F was fused to gene encoding maltose binding protein and was expressed in *E. coli* (Holley et al., 2001). The produced fragment was immunogenic in mice and protected against challenges with BoNT/F. As an alternative approach to designing and preparing synthetic genes, the elevated expression of certain rare tRNAs was reported to improve production of clostridial proteins in *E. coli* (Zdanovsky and Zdanovskaia, 2000). Of the three tRNAs utilized, ATA (*ileX*), AGA (*ArgU*), and CTA (*leuW*), ATA was most important for increased synthesis. However, it was unclear whether biological activity was recovered to a degree comparable to the H and L chains produced by purification and biochemical separation of the chains (Sathyamoorthy and DasGupta, 1985). Thus, a major concern of clostridial toxin fragments produced in heterologous systems is whether the resulting proteins can be recovered with full retention of biological activity. They have certainly been shown to retain epitopes sufficient for immunostimulation in mice and protection against NT challenge, but this is not a highly refined evaluation of biological activity. To our knowledge, NT fragments produced in heterologous hosts followed by attempts to reconstitute activity have met with only partial success. For example, reconstitution of recombinant H and L of TeTx chains had 300-fold less mouse lethality than native TeTX, while reconstituted native separated chains had only 7-fold less mouse lethality than purified native TeTX (Li et al., 1999). The recovery of full biological activity appears to be a significant challenge for both recombinant and biochemically separated fragments followed by reconstitution. Recently, truncated forms of the light chains of BoNT and TeNT have been expressed in *E. coli* as fusion proteins (Kadkhodayan et al., 2000; Tonello et al., 1999), and they appear to have catalytic activity and have been crystallized for structural determination.

10. Evidence for lateral gene transfer of BoNT and other clostridial genes and evolution of toxigenesis

An intriguing and important genetic property of certain toxin genes in clostridia is that they are often associated with genetic elements that can be laterally transferred across phylogenetic barriers, occasionally to nontoxigenic clostridia. The possibility for transfer of BoNT genes was first suspected in the investigation of infant botulism cases in Italy and in the USA, when strains of *C. butyricum* or *C. baratii* were recovered from infants with botulism (Aureli et al., 1986; Hall et al., 1985; McCroskey et al., 1986, 1991). Characterization of the BoNTs produced by these strains showed that they were very similar to type E and F botulinum toxins by biochemical, immunological, and genetic properties. The mechanism by which they were transferred to the nontoxigenic clostridia has not been elucidated, and both plasmid-mediated (Hauser et al., 1995) and phage-mediated transfer (Zhou et al., 1993) were suggested. The recent isolation of several strains of *C. butyricum* in Japan that produce type E-like botulinum toxins support a phage-mediated transfer since the NT genes are located on the chromosome as determined by Southern hybridizations (Meng et al., 1997).

BoNTs are among the largest protein toxins and various approaches have shown that they consist of three primary domains that differ in amino acid sequence and secondary and tertiary architectures and contain novel protein folds and catalytic activities (Lacy et al., 1998; Schiavo et al., 2000; Swaminathan and Eswaramoorthy, 2000). Structural studies support that BoNTs evolved by the assembly of nonhomologous genes, and it is likely that the individual protein domains also evolved by the assembly and exchange of small gene segments. The assembled chimeric protein structure acquired an array of functions including resistance to proteolysis in the gut, neurospecific binding, ability to translocate to nerve cytosol, and the ability to catalytically cleave nerve proteins involved in exocytosis. Tracing the evolution of BoNTs is a formidable task but is becoming more approachable now that the structure and function of this family of proteins is being revealed (Lacy et al., 1998; Schiavo et al., 2000; Swaminathan and Eswaramoorthy, 2000). One experimental approach to dissect the evolution of structure/function of NTs is by

the combinatorial shuffling of nonhomologous peptide segments in vitro (Reichmann and Winter, 2000). In theory, it would be possible to select for functions including resistance to proteolysis, binding to nerves, transport through membranes, and catalytic cleavage of neuronal protein substrates. The selection for binding to protein folds could also be approached by affinity binding to monoclonal antibody fragments or other binding ligands produced through phage display (Sidhu et al., 2000) or other methods. The sequences and functional properties could be sequentially examined and the process of 'toxin selection' reiterated.

Evidence indicates that the genome of neurotoxigenic clostridia exists in a dynamic state, and that moveable genetic elements are involved in rearrangement and recombination of specific regions. Such plasticity has been revealed in several bacterial genera (Koltsø, 1997). Further development of genetic tools to study the toxigenic clostridia, combined with physical mapping and sequencing of clostridial genomes, will provide insights into the biology and evolution and function of toxigenesis in the neurotoxigenic clostridia.

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